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Differential gene expression pattern in brains of acrylamide-administered mice

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Abstract : The present study was performed to evaluate the relationship between the neurotoxicity of acrylamide and the differential gene expression pattern in mice. Both locomotor test and rota-rod test showed that the group treated with higher than 30 mg/kg/day of acrylamide caused impaired motor activity in mice. Based on cDNA microarray analysis of mouse brain, myelin basic protein gene, kinesin family member 5B gene, and fibroblast growth factor (FGF) 1 and its receptor genes were down-regulated by acrylamide. The genes are known to be essential for neurofilament synthesis, axonal transport, and neuro-protection, respectively. Interestingly, both FGF 1 and its receptor genes were down-regulated. Genes involved in nucleic acid binding such as AU RNA binding protein/enoyl-coA hydratase, translation initiation factor (TIF) 2 alpha kinase 4, activating transcription factor 2, and U2AF 1 related sequence 1 genes were down-regulated. More interesting finding was that genes of both catalytic and regulatory subunit of protein phosphatases which are important for signal transduction pathways were down-regulated. Here, we propose that acrylamide induces neurotoxicity by regulation of genes associated with neurofilament synthesis, axonal transport, neuro-protection, and signal transduction pathways.

Keywords : acrylamide, cDNA microarray, locomotor test, neurotoxicity, rota-rod test

Introduction

Exposure to acrylamide is a growing concern because acrylamide is abundant in heat-treated food products [6]. Even though it is not detected before cooking, acrylamide is formed as a byproduct during the high-temperature cooking processes [13]. Previous studies observed the effects of acrylamide on neurotoxicity. Acrylamide intoxication produces peripheral neuropathy characterized by weakness and ataxia of hind limb in both humans and experimental animals [5]. Pathological changes such as axonal swelling in junctional folds and intramuscular nerves, which results in Wallerian-like degeneration [5]. It affects the peripheral nervous systems as well as the central nervous system presented as central-peripheral distal axonopathy [12]. Nerve terminals, not axons, are the primary sites of acrylamide action which are responsible for the autonomic, sensory, and motor defects that accompany acrylamide intoxication [10]. Spatiotemporal analysis suggested that acrylamide intoxication produced selective nerve terminal degeneration, and the degeneration moved as a function of time in a somal direction along the corresponding axon in brainstem [8], or in forebrain [7]. Because cerebellar coordination of somatomotor activity is mediated solely through efferent projections of the Purkinje cell, injury to this neuron may contribute to gait abnormalities that characterize acrylamide neurotoxicity [9]. Neurotoxicity of acrylamide was represented as the reduced motor activities of mice in both locomotor activity test and rota-rod test [4, 15]. Even though many previous studies observed the neurotoxicity of acrylamide, the basic mechanisms of the toxicity were not explained thoroughly.

We hypothesized that the neurotoxicity of acrylamide might result from up/down-regulation of genes involved in neuronal signaling pathways. The purpose of this study was to evaluate the relationship between the neurotoxicity of acrylamide and the differential gene expression pattern in mice brains. To evaluate this, we performed cDNA microarray analysis with brains isolated

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from mice treated with acrylamide. The present study will give the valuable information for the detailed mechanisms of the acrylamide toxicity based on the function of the genes.

Materials and Methods

Animals and administration of acrylamide

Adult male ICR mice were purchased from Charles River Technology (Korea). The mice, aged 45~50 days and weighing 25~30 g, were divided into six groups of 8 animals each. The animals were housed 4 per polycarbonate cage with wood shavings as bedding, and were maintained under a controlled environment with temperature at 23 ± 2°C, relative humidity at 55 ± 5%, and a 12 h/12 h light/dark cycle throughout the experimental period. Acrylamide was administered to each group of mice at doses of 0, 5, 15, 30, 45, and 60 mg/kg/day for 10 consecutive days by oral gavage at a dosing volume of 20 mL/kg. After 24 h of last administration, each group of mice was subjected to either locomotor activity test or rota-rod test. After the tests, the mice were sacrificed by decapitation. Brains were isolated from each group of 8 mice treated with acrylamide at doses of 0 or 60 mg/kg/day, and stored in RNA later (Sigma-Aldrich, USA) at –20°C until use for cDNA microarray analysis.

Locomotor activity test

After 24 h of last administration, each group of mice was subjected to locomotor activity test. Traveled (ambulatory) distances were measured using a locomotor activity cage with 43 cm square and 30 cm height walls (MED-associates, USA). The activity cage is equipped with 16 infrared photocells on the wall. Activity was expressed as the distance moved by calculating based on the number of interruptions of the photobeams, during 3 min after 60 min habituation period. For positive control, chlorpromazine HCl (5 mg/kg; Sigma-Aldrich, USA) was injected intramuscularly, and physiological saline solution (Choong-wae Pharm., Korea) was injected for negative control.

Rota-rod test

After 24 h of last administration, each group of mice was subjected to rota-rod test. The effect of acrylamide on motor function was observed using rota-rod tester (Dae-Jong, Korea) consisting of a rotating spindle (12 cm width; 6 cm diameter) and individual compartments for each mouse. For training, the animals were placed on the rota-rod at 4 rpm for about 10 min. After 10 min adaptation, the duration of riding on a rotating spindle was measured at 4 rpm for 300 sec. When the duration of riding exceeded 300 sec, the mouse was removed from the rod, and the riding time was recorded as 300 sec. Chlorpromazine HCl (5 mg/kg; Sigma-Aldrich, USA) was injected to a positive control group and physiological saline solution (Choong-wae Pharm., Korea) was injected to a negative control group.

Microarray analysis

For mouse brain, cDNA microarray analysis was performed using Platinum Biochip Mouse 8.0K (GenoCheck, Korea) that includes 8,000 mouse cDNAs from Incyte Genomics (Palo Alto, USA). Total RNA was extracted from testes of acrylamide treated group (60 mg/kg/day, 5 days) and untreated group according to the manufacture's instructions (GenoCheck, Korea). Fluorescence-labeled cDNA probes were prepared from 30 μg of total RNA by oligo $(dT)_{18}$-primed polymerization using SuperScript II reverse transcriptase (Gibco BRL, USA) in a total reaction volume of 30 μL. The reverse transcription mixture included 400 U Superscript RNase H-reverse transcriptase (Gibco BRL, USA), 0.5 mM dATP, dTTP, dGTP, 0.2 mM dCTP, and 0.1 mM Cy3 or Cy5 labeled dCTP (NEN). After reverse transcription, the sample RNA was degraded by adding 5 μL of stop solution (0.5 M NaOH/50 mM EDTA), and incubated at 65°C for 10 min. The labeled cDNA mixture was then concentrated using ethanol precipitation method. The concentrated Cy3 or Cy5 labeled cDNAs were resuspended in 12 μL of hybridization solution consisting of 2× Denhardt's solution, 4.5% SDS, 1× SSC, 1 mM EDTA, 0.25 M Na_2HPO_4, and 0.05 mg/mL yeast tRNA. Labeled cDNAs was denaturized at 95°C for 2 min, and incubated in 45°C water chamber for 20 min, then placed on the spotted slide position and covered by a cover slip (22 mm × 22 mm). The slides were hybridized for 14 h in 62°C hybridization chamber. The hybridized slides were washed in 2× SSC, 0.1% SDS for 2 min, 1× SSC for 3 min, and then 0.2× SSC for 2 min at room temperature. The slides were centrifuged at 1,000 rpm for 2 min to dry.

The hybridized slides were scanned with GenePix scanner (4000B; Axon Instruments, USA), and the scanned images were analyzed using the software

programs GenePix Pro (ver. 4.0; Axon Instruments, USA) and GeneSpring (ver. 5.0; Silicon Genetics, USA). To normalize for fluorophore-specific variation, control spots containing housekeeping genes (GAPDH and β-actin) were applied to each quadrant during the arraying process. These elements were quantitated, and the ratios of the signals were determined for normalization. To determine the background signal intensity, we spotted the yeast DNA on each slide. Values at least 1.5 fold above the background intensity were considered significant. To filter out the unreliable data, spots with signal-to-noise ratio below 3 were not included in the data. The results are analyzed with the Tree View program (microarray software, USA).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using SPSS software for Windows. When the ANOVA test yielded statistical differences ($p < 0.05$ or 0.01), Dunnett's two-tailed *t*-test was used to compare each acrylamide-treated group against the control group. The value of $p < 0.05$ was used as the criterion for statistical significance. All data were expressed as means ± SE.

Results

Locomotor activities, assessed by traveled distances, monitored in mice treated with acrylamide are illustrated in Fig. 1. The activity was reduced significantly in the group treated with 30 mg/kg/day of acrylamide ($p < 0.05$). The traveled distance was further reduced in the group treated with higher than 45 mg/kg/day of acrylamide ($p < 0.001$). The activity was almost abolished in the group treated with chlorpromazine HCl (5 mg/kg). The riding time was also estimated on the rota-rod in its rotation speed at 4 rpm (Fig. 2). The values were not changed significantly in groups treated with less than 15 mg/kg/day of acrylamide. In contrast, complete failures of the test were observed in the groups treated with higher than 30 mg/kg of acrylamide ($p < 0.001$). The values of these groups were even lower than that of positive control group treated with chlorpromazine HCl (5 mg/kg).

To observe the genes related to neurotoxicity in mice, microarray analysis was performed with brain isolated from acrylamide-treated mice. Table 1 summarizes up/down-regulated genes in mouse brain by acrylamide.

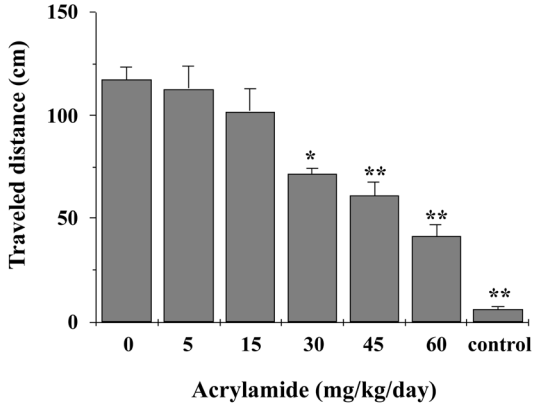

Fig. 1. Locomotor activity of mice treated with acrylamide. Locomotor activity was measured as described in Materials and Methods. Acrylamide was administered orally to the group of mice for 10 days. Tests were performed at 24 h after the last administration. For positive control, chlorpromazine HCl was injected intramuscularly. Each value shows the mean (± SE) of total traveled distance (cm). Asterisk indicates a significant difference from vehicle group, $*p < 0.05$; $**p < 0.001$.

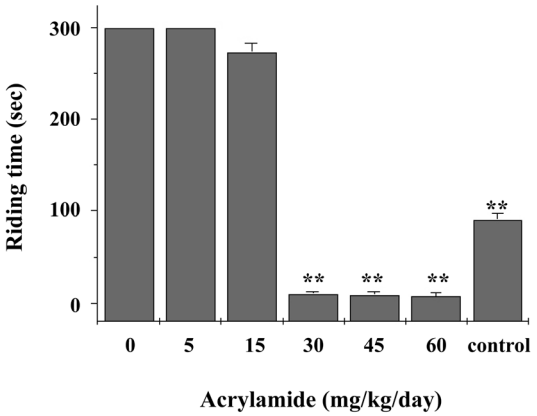

Fig. 2. Rota-rod test (riding time) of mice treated with acrylamide. Rota-rod test was measured as described in Materials and Methods. Mice were administrated with acrylamide orally for 10 days. Rota-rod test was preformed when 24 h had been passed after the last administration. Chlorpromazine HCl (5 mg/kg) was injected to a positive control group. Each value shows the mean (± SE) of riding time (sec). Asterisk indicates a significant difference from vehicle group, $**p < 0.001$.

Genes related to neurons, cell growth, cell proliferation, nucleic acid binding, and dephosphorylation were up/down-regulated. In association with neurons, both myelin basic protein and kinesin family member 5B

Table 1. Up/down-regulated genes in mouse brain by acrylamide

Function	Accession No.	Gene name	Fold changed
Neuron-related	AA059540	Myelin basic protein	0.63
	AI508643	Kinesin family member 5B	0.44
Cell growth	AA108370	glutathione S-transferase, pi 2	1.57
	AA542013	Fibroblast growth factor (FGF) receptor 1	0.66
	AA261582	Fibroblast growth factor (FGF) 1	0.65
	AA108848	cysteine-rich with EGF-like domains 1	0.63
Cell proliferation	AA051654	metallothionein 1	1.63
	W16205	B-cell translocation gene 2	1.58
	W97212	Son cell proliferation protein	0.60
Nucleic acid binding	AI893340	AU RNA binding protein/enoyl-coA hydratase	0.67
	AA016507	translation initiation factor (TIF) 2 alpha kinase 4	0.60
	W34018	hypothetical protein D330027P03	0.51
	AA414544	activating transcription factor 2	0.42
	AA274915	U2AF 1, related sequence 1	0.41
Dephosphorylation	AA198158	protein phosphatase 5, catalytic subunit	0.67
	W65520	protein phosphatase 1, regulatory subunit 1C	0.64
	W81953	protein tyrosine phosphatase, receptor type, F	0.63
	AA087123	protein phosphatase 2A, regulatory subunit B	0.35

genes were down-regulated. In association with cell growth and proliferation, glutathione S-transferase pi2, metallothionein 1, and B-cell translocation gene 2 genes were up-regulated whereas cystein-rich with EGF-like domain 1 and Son cell proliferation protein genes were down-regulated. Interestingly, both fibroblast growth factor (FGF) 1 and its receptor genes were down-regulated. Genes involved in nucleic acid binding such as AU RNA binding protein/enoyl-coA hydratase, translation initiation factor (TIF) 2 alpha kinase 4, activating transcription factor 2, and U2AF 1 related sequence 1 genes were down-regulated. More interesting finding was that genes of both catalytic and regulatory subunit of protein phosphatases were down-regulated (Table 1).

Discussion

The purpose of this study was to evaluate the relationship between the neurotoxicity of acrylamide and the differential gene expression pattern in mice brains using cDNA microarray analysis. In the present study, we observed that genes associated with several functions were regulated in mice brains by acrylamide. Especially, the regulation of genes associated with neurofilament synthesis, axonal transport and neuro-protection might be related to the neurotoxicity of acrylamide.

In association with neurons, myelin basic protein gene which is required for the myelin compaction was down-regulated in mouse brain by acrylamide. Myelin basic protein is an essential component for normal myelin compaction which is implicated in human demyelinating diseases [2]. Previous study observed a significant reduction of mean cross area of myelinated nerve fibers in rats exposed to acrylamide, which suggests that the neuronal atrophy might represent a defect of neurofilament synthesis [17]. Therefore, down-regulated myelin basic protein gene observed in this study might result in a poor compaction of myelin in nerve fibers, which might be a factor for neurotoxicity of acrylamide.

Kinesin gene in the brain of the acrylamide-treated mouse was also down-regulated. It was reported that kinesin and microtubules are covalently modified by acrylamide resulting in reduced affinity each other, and this was observed as a mechanistic factor in axonal degeneration [14]. Such mechanisms also seem to be related to chromosomal genotoxicity by affecting the process of chromosomal segregation [1]. Furthermore, it was reported that potential mechanisms of acrylamide neurotoxicity include enhanced calcium entry [11] and interference with axonal transport, particularly affecting microtubules and kinesin [14, 16]. Therefore, it is possible that the reduced level of kinesin might be a factor for interfering axonal transport. The results

suggest that neurotoxicity of acrylamide might result from not only covalent modification of kinesin but also down-regulated kinesin gene by acrylamide.

FGF 1 is an endothelial cell mitogen and serves as a mitogen and/or differentiating factor that can be neuro-protective effect for other cell types within the CNS [3]. In the present study, down-regulation of FGF 1 gene might indirectly affects neuro-protective effect within the CNS that might be a factor for neurotoxicity of acrylamide. Interestingly, genes of both catalytic and regulatory subunit of protein phosphatases were down-regulated. Down-regulation of these genes might perturb the balance of phosphorylation/dephosphorylation of proteins that is the essential procedure for signal transduction pathways. Therefore, the disturbed level of genes related to neuronal cells or signals might explain the impaired and decreased motor activity of acrylamide-treated mice.

In this study, we observed that genes associated with several functions were regulated by acrylamide. Especially, the regulations of genes involved in neurofilament synthesis, axonal transport, and neuro-protection were observed in brains isolated from acrylamide-treated mice. We propose that acrylamide induces neurotoxicity by the regulation of genes associated with neurofilament synthesis, axonal transport, and neuro-protection. Though it is primitive, the present study will give the valuable information for the future studies on the detailed mechanisms of acrylamide toxicity based on the function of the genes.

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